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# ONCOLOGY

# Analysis of Bone Tissue Condition in Patients with Diffuse Large B-Cell Lymphoma without Bone Marrow Involvement

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We studied changes in the bone tissue in patients with diffuse large B-cell lymphoma at the onset of the disease (*N*=41; before chemotherapy) and 5-16 years after the end of treatment (*N*=47). Osteodensitometry, biochemical markers of osteoporosis in the blood and urine, and gene expression in multipotent mesenchymal stromal cells were analyzed. In multipotent mesenchymal stromal cells of all patients, the expression of genes associated with bone and cartilage differentiation (*FGF2*, *FGFR1*, *FGFR2*, *BGLAP*, *SPP1*, *TGFB1*, and *SOX9*) was changed. In primary patients, the ratio of deoxypyridinoline/creatinine in the urine and blood level of β-cross-laps were increased, while plasma concentration of vitamin D was reduced, which indicates activation of bone resorption. No differences between the groups were revealed by osteodensitometry. No direct relationship between changes in gene expression in multipotent mesenchymal stromal cells and osteoporosis markers was found. The presence of a tumor in the body affects the bone marrow stroma, but achievement of remission and compensatory mechanisms provide age-appropriate condition of the bone tissue.

**Key Words:** *diffuse large B-cell lymphoma; multipotent mesenchymal stromal cells; bone mineral density; markers of bone metabolism; gene expression*

The bone marrow harbors two types of stem cells: hemopoietic and stromal. Mesenchymal stem cells are the precursors of all lines of differentiation of the bone marrow stroma regulating hemopoiesis. Multipotent mesenchymal stromal cells (MMSC) are descendants of a stem cell identified in culture; these plastic-adherent cells can differentiate into osteogenic, chondrogenic, and adipogenic lineage cells [4]. MMSC are involved in the formation and remodeling of the bone tissue.

In 10-25% patients with diffuse large B-cell lymphoma (DLBCL), bone marrow involvement is detected using histological or molecular methods [11]. In the remaining patients with DLBCL, the bone marrow is believed to be not involved in the tumor process. It could be assumed that stromal bone marrow precursor cells in this group of patients are unchanged, because the direct influence of tumor cells is excluded. It is known that tumor cells adapt the bone marrow stromal microenvironment in leukemia. The effect of tumor cells can be directly transmitted by the paracrine route or through exosomes secreted by tumor cells [7]. However, previous studies showed that MMSC of patients with DLBCL without bone marrow involvement are altered [5]. Changes in the expression of some genes in MSC of DLBCL patients suggested the presence of changes in the bone tissue of these patients.

The condition of the bone tissue depends on phosphorus-calcium metabolism, parathyroid hormone, vi-

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tamin D, growth hormone, calcitonin, other hormones [3]. Cytostatic drugs and long-term intake of glucocorticosteroids also affect the process of bone resorption and formation [2]. In this study, an attempt was made to analyze osteoporosis indicators in patients with DLBCL without bone marrow involvement before therapy and in patients who completed chemotherapy more than 5 years ago. In particular, we evaluated bone mineral density (BMD), biochemical markers of bone tissue metabolism in the blood, and gene expression associated with osteogenesis in MMSC.

Our aim was to study the association between characteristics of MMSC of patients and condition of the bone tissue at the onset of the disease and after therapy.

## MATERIALS AND METHODS

All patients were diagnosed according to the WHO criteria (2008, 2017) for tumors of hemopoietic and lymphoid tissue [11]; the diagnosis was confirmed by histological and immunohistochemical studies of the tumor. MMSC were studied in 41 patients at the onset of the disease (3 men and 38 women, median age 51 years) and 47 patients who completed treatment more than 5 years ago with ongoing remission of the disease (26 men and 21 women, median age 61 years). The control group consisted of 31 bone marrow donors (15 men and 16 women, median age 53 years). All people included in the study signed an informed consent to participate in the study. All patients underwent 4-6 courses of chemotherapy. Remission of the disease was confirmed by computed tomography or positron emission tomography (PET-CT).

MMSC were isolated from 3-5 ml of the bone marrow obtained during diagnostic puncture in patients or exfusion from donors (informed consent was obtained from all participants). MMSC were isolated and cultured as described elsewhere [5].

The level of gene expression in MMSC was determined at the first passage by RT-PCR (Taq-Man modification) on an Abiprism 7500 instrument (Applied Biosystems). RNA was isolated using a standard protocol. Reverse transcriptase (M-MLV, Promega) was used to construct the first DNA strands after hybridization of mRNA with a mixture of poly-T primers and random hexamers. The housekeeping genes *GAPDH* and *BACT* (β-actin) were used for standardization of the samples, and the relative level of gene expression was calculated using ΔΔCt [8].

A comprehensive examination of the bone tissue was carried out in 49 DLBCL patients: 11 patients before the start of therapy and 24 patients who completed the therapy more than 5 years ago. Age-matched healthy subjects comprised the control group ($N$=14). PET-CT was performed in all patients prior to densitometry to determine the volume of the tumor lesion or to confirm remission of the disease. Densitometry was performed on a clinical bone dual energy X-ray osteodensitometer Discovery A (Hologic) by comparing measured values with the maximum values characteristic of people of the corresponding age expressed in standard deviations (SD, or T-, Z-scale). Deviation <1 SD was considered normal, values with deviation less than 1 SD, but more than -2.5 SD were classified as osteopenia, and less than -2.5 SD as osteoporosis [1]. Osteodensitometry was performed at standard points for which reference normative values are known (lumbar vertebrae L1-L4 and the femoral neck). Biochemical markers of osteoporosis have been tested in the blood (osteocalcin, β-cross-laps, parathyroid hormone, and 25-OH vitamin $D_3$) and in the urine (urinalysis and deoxypyridinoline (DPD) level).

Statistical analysis was performed by unpaired Student's $t$ test, multiple comparison, and ANOVA using GraphPad Prism 8.1 (GraphPad Software Inc). The differences were considered significant at $p<0.05$.

## RESULTS

In MMSC from patients, the expression of genes involved in bone differentiation was significantly changed. In primary patients, the expression of *FGF2* and receptor for this growth factor (*FGFR2*) was increased by 1.8 and 1.6 times, respectively (Table 1). Expression levels of genes directly involved in the formation of the bone tissue *BGLAP* (osteocalcin), *SPP1* (osteopontin), *BMP2*, and *BMP4* were not changed. Many years after the end of treatment, the relative expression levels of *TGFB1*, *SOX9*, *FGFR1*, *VEGF*, and *BMP2* in patients were significantly reduced, while the levels of *SPP1* and *FGFR2* were increased in comparison with those in donors. All these genes are either directly or indirectly involved in remodeling and maintenance of the bone tissue. In patients with remission, the relative expression levels of *FGF2*, *FGFR1*, *VEGF*, *BGLAP*, and *BMP2* were significantly reduced in comparison with primary patients.

These data served as the starting point for the study of the bone tissue of patients with DLBCL. Markers of bone resorption, in particular, the DPD/creatinine ratio in the urine and β-cross-laps in the blood, were significantly increased in the debut of the disease (Fig. 1, Table 2). At the same time, in patients at the onset of the disease, the level of vitamin D3 was reduced in comparison with the control group.

Despite clear-cut changes at the cellular and molecular level, densitometry revealed no differences in the bone density between the patients with DLBCL and the control group (Fig. 2). The frequency of os-

**TABLE 1.** Relative Gene Expression in MMSC from Donors and DLBCL Patients

| Gene | Control group | Onset of the disease | Remission (5 years after treatment) |
|---|---|---|---|
| MMP2 | 3.92±0.37 | 3.48±0.34* | 2.57±0.21*+ |
| IL6 | 11.25±3.10 | 15.18±2.80* | 7.30±0.92*+ |
| TGFB1 | 1.12±0.07 | 1.08±0.06 | 0.93±0.06* |
| PDGFRb | 0.82±0.07 | 0.7±0.05 | 0.84±0.07 |
| PPARG | 1.54±0.15 | 1.69±0.13 | 1.96±0.22 |
| SOX9 | 1.47±0.20 | 1.17±0.12 | 0.94±0.10* |
| FGF2 | 5.12±0.65 | 9.03±1.55* | 4.40±0.34+ |
| FGFR1 | 0.96±0.07 | 0.79±0.05 | 0.60±0.04*+ |
| FGFR2 | 2.21±0.24 | 3.62±0.28* | 3.97±0.32* |
| VEGF | 0.22±0.04 | 0.22±0.03 | 0.13±0.01*+ |
| SPP1 | 0.07±0.01 | 0.11±0.02 | 0.18±0.05* |
| ICAM1 | 1.18±0.22 | 1.02±0.22 | 0.48±0.05*+ |
| BGLAP | 2.04±0.74 | 1.74±0.20 | 0.94±0.08+ |
| SMURF1 | 3.72±0.24 | 3.42±0.19 | 3.88±0.31 |
| BMP4 | 1.19±0.18 | 0.81±0.09 | 1.21±0.18 |
| BMP2 | 1.20±0.36 | 1.18±0.22 | 0.44±0.07*+ |
| IL8 | 0.20±0.06 | 2.38±0.84* | 0.13±0.03+ |
| MCAM | 5.30±0.85 | 6.93±0.89* | 5.88±0.57 |

**Note.** $p<0.05$ in comparison with *donors, +onset of the disease.

teopenia and osteoporosis was not increased in the studied groups.

Examination of patients in remission after the end of treatment showed that all biochemical parameters and bone density were within the normal range. However, MMSC of these patients differed from cells of the control group. This was manifested in a significant decrease in the relative expression of genes encod-

**TABLE 2.** Biochemical Parameters of DLBCL Patients

| Parameter | Control group | Onset of the disease | Remission (5 years after treatment) |
|---|---|---|---|
| DPD/creatinine in the urine | 6.41±0.52 | 12.7±1.9* | 5.44±0.26 |
| DPD | 66.50±8.79 | 193.9±58.8* | 72.95±7.47 |
| Vitamin D | 21.35±2.21 | 12.7±2.1* | 18.28±1.52 |
| Parathormone | 2.9±0.55 | 3.9±1.0 | 4.21±0.56 |
| Osteocalcin | 19.71±1.79 | 15.8±2.2 | 17.33±1.18 |
| β-cross-laps | 0.44±0.04 | 0.7±0.1* | 0.41±0.03 |
| Total protein | 73.29±1.16 | 69.5±3.4 | 74.55±0.76 |
| Albumin | 43.38±0.40 | 39.0±2.1 | 43.03±0.46 |
| Globulin | 30.08±0.99 | 30.5±2.2 | 30.78±0.86 |
| Calcium | 2.41±0.03 | 2.5±0.1 | 2.40±0.02 |
| Phosphorus | 1.04±0.03 | 1.1±0.0 | 1.01±0.03 |
| Alkaline phosphate | 77.99±6.47 | 148.5±68.4 | 86.68±8.34 |
| $Ca^{2+}$ | 1.17±0.02 | 1.2±0.0 | 1.14±0.01 |
| Creatinine | 69.72±3.02 | 63.4±3.1 | 84.51±4.97 |
| Urea | 5.63±0.31 | 4.9±0.5 | 6.27±0.49 |

**Note.** *$p<0.05$ in comparison with the control.

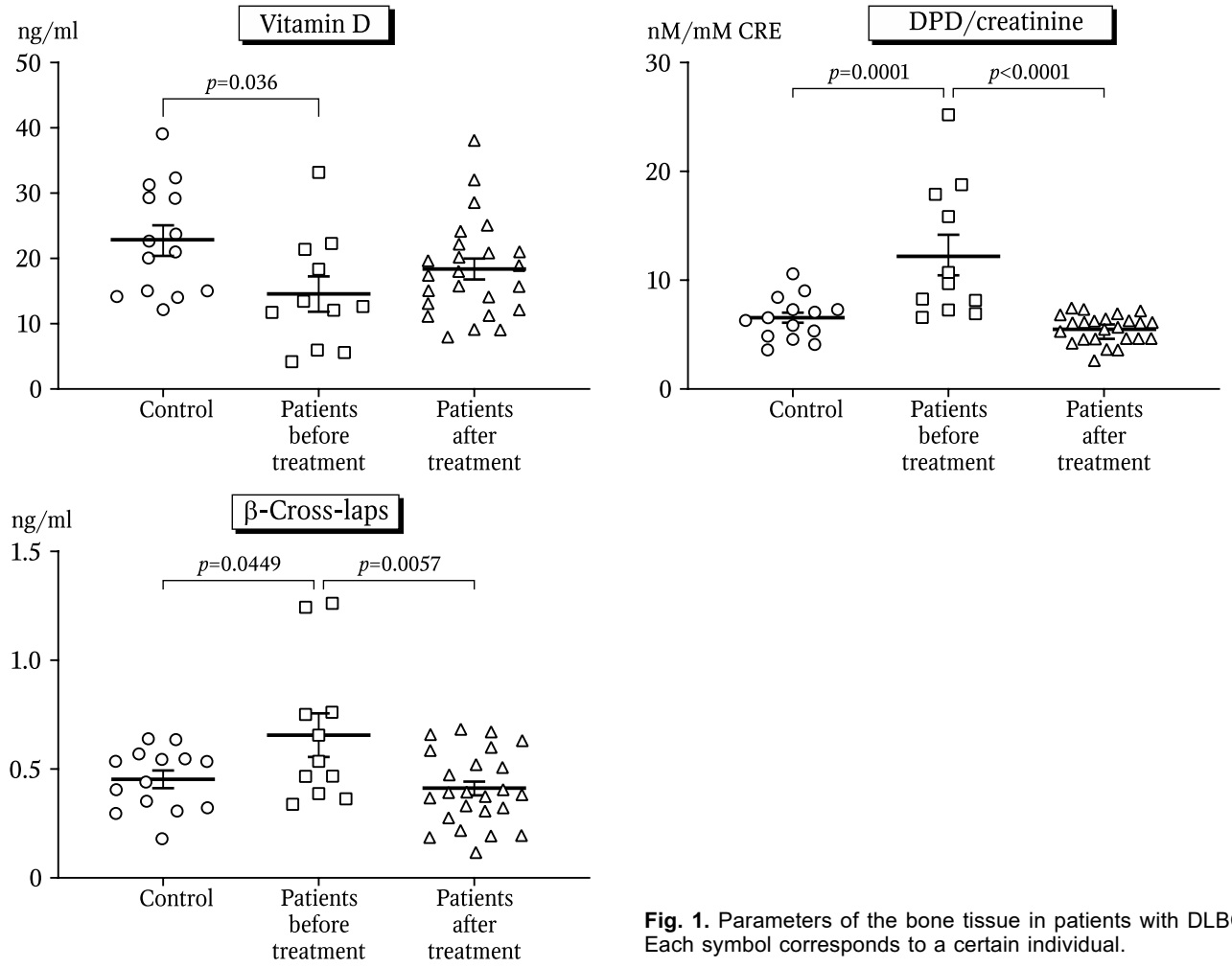


**Fig. 1.** Parameters of the bone tissue in patients with DLBCL. Each symbol corresponds to a certain individual.

ing proinflammatory cytokines IL-6 and IL-8, factors involved in bone formation osteocalcin (*BGLAP*) and *BMP2* and cartilage formation (*SOX9*), and proteins associated with MMSC proliferation (*FGF2* and its receptor *FGFR1*).

Bone-contacting tumor cells are known to modulate the expression of many genes and proteins, including MMPs, TGFβ, IL6, JAG1, GLI2, RUNX2, HIF1α, PTHRP, and CaSR [12]. In patients included in the study, tumor cells did not directly contact with the bone tissue, though expression of many genes involved in bone formation and inflammatory processes in MMSC obtained from their bone marrow was changed. It turned out that at the onset of the disease, only three parameters were changed in the blood and urine of patients: DPD/creatinine ratio in the urine and the levels of β-cross-laps and vitamin D in the blood. DPD is a molecule that forms cross-links between collagen subunits and β-cross-laps are fragments of collagen degradation. Increase blood concentrations of these markers in patients at the onset of the disease indicate enhanced bone resorption. Osteoclasts, bone tissue macrophages responsible for bone resorption, are activated by many cytokines and chemokines, in particular IL-6 and IL-8, the expression of which is increased in MMSC from DLBCL patients (Table 1). It should be borne in mind that osteoclasts interact with osteoblasts, descendants of MMSC, in the bone marrow. This interaction underlies the process of bone remodeling [6]. Markers of bone differentiation of MMSC were not changed in patients at the onset of the disease, but significantly differ from the control group in patients with remission. The expression of the terminal bone differentiation marker *BGLAP* gene (osteocalcin) was significantly reduced, but blood concentrations of osteocalcin remained within the normal range. The marker of early bone differentiation *SPP1* (osteopontin) significantly increased in MMSC from patients in many years after the end of treatment. The marker of cartilage differentiation *SOX9* tended to decrease in patients at the onset of the disease and was significantly reduced in 5 years after the end of treatment. Vitamin D is necessary for calcium absorption in the intestine, normal osteogenesis, and bone

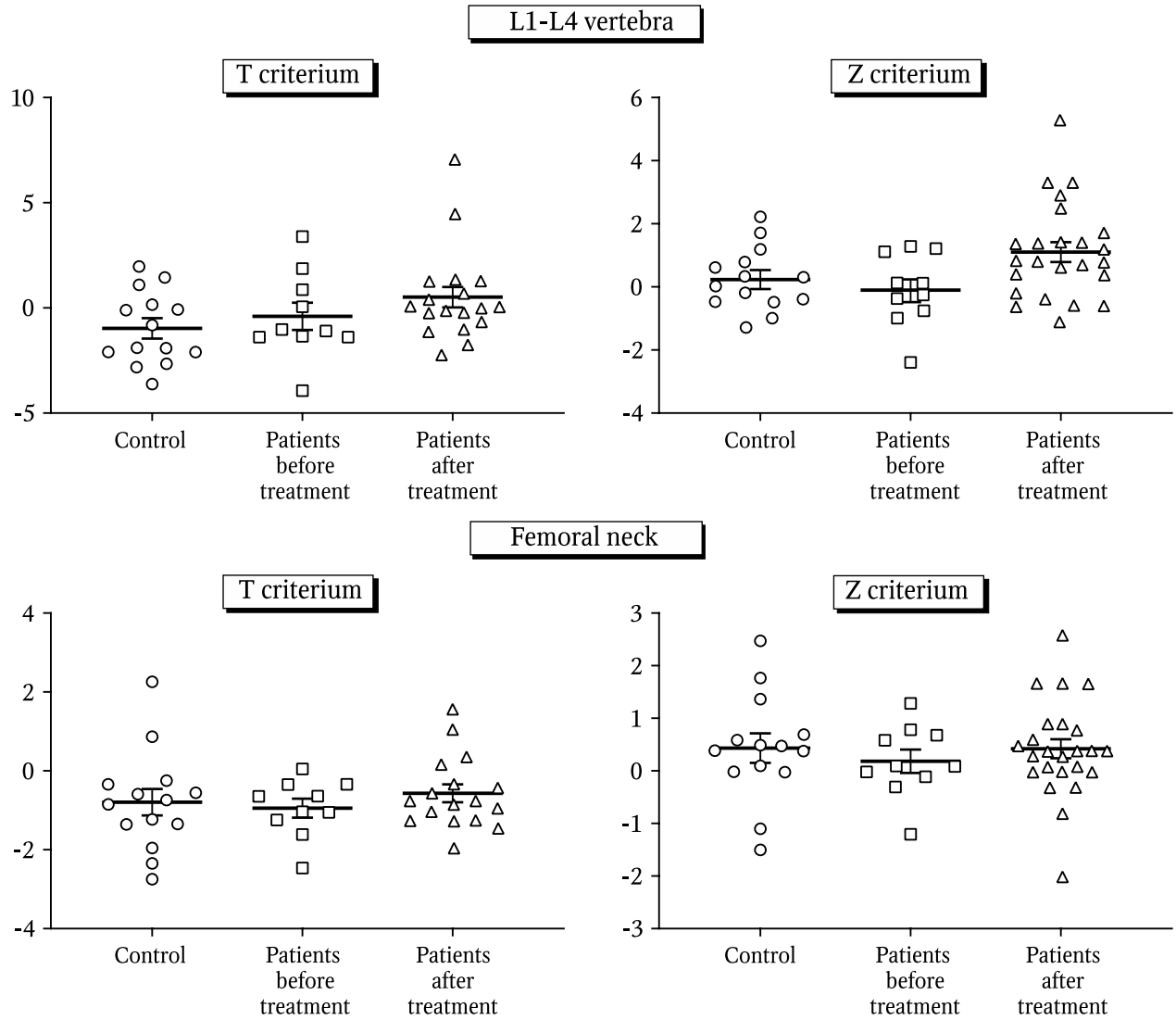


**Fig. 2.** Analysis of T and Z criteria in the spine and femoral neck of DLBCL patients. Each symbol corresponds to a certain individual.

mineralization. The observed decrease in its level in the blood can be associated with compensatory activation of all these processes. No direct correlations between changes in gene expression in MMSC and biochemical parameters were found. However, these multidirectional changes indicate restructuring of bone metabolism.

Densitometry of the spine and femoral neck revealed no differences between the groups. The absence of manifest skeletal disorders can be explained by long period of bone tissue renewal, though signs of increased bone resorption are clearly seen in biochemical blood and urine tests. In patients in remission examined 5 years after the end of treatment, the biochemical parameters of bone metabolism without influences from the tumor are normalized.

The detected changes in MMSC cultures are confirmed by biochemical markers of enhanced bone resorption at the onset of the disease. However, complex and long-term processes associated with the regulation of whole-body homeostasis did not allow us to establish a direct association between the characteristics of MMSC from DLBCL patients and the state of the bone tissue at the onset of the disease and at delayed terms after treatment. This should be taken into account in the complex analysis of progenitor cells in pathology.

Thus, the presence of a tumor in the body affects the stroma of the bone marrow, but achieving long-term remission and compensatory mechanisms provide age-appropriate condition of the bone tissue.

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